Attenuation of Angiotensin II-Induced Hypertension in BubR1 Low‐Expression Mice Via Repression of Angiotensin II Receptor 1 Overexpression

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Attenuation of Angiotensin II–Induced Hypertension in BubR1 Low-Expression Mice Via Repression of Angiotensin II Receptor 1 Overexpression

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Background—Angiotensin II (Ang II) can cause hypertension and tissue impairment via AGTR1 (Ang II receptor type 1), particularly in renal proximal tubule cells, and can cause DNA damage in renal cells via nicotinamide adenine dinucleotide phosphate oxidase. BubR1 (budding uninhibited by benzimidazole-related 1) is a multifaceted kinase that functions as a mitotic checkpoint. BubR1 expression can be induced by Ang II in smooth muscle cells in vitro, but the relationship between systemic BubR1 expression and the Ang II response is unclear.

Methods and Results—Twenty 24-week-old male BubR1 low-expression mice (BubR1$^{L/L}$ mice) and age-matched BubR1$^{+/+}$ mice were used in this study. We investigated how Ang II stimulation affects BubR1$^{L/L}$ mice. The elevated systolic blood pressure caused by Ang II stimulation in BubR1$^{+/+}$ mice was significantly attenuated in BubR1$^{L/L}$ mice. Additionally, an attenuated level of Ang II–induced perivascular fibrosis was observed in the kidneys of BubR1$^{L/L}$ mice. Immunohistochemistry revealed that the overexpression of AGTR1 induced by Ang II stimulation was repressed in BubR1$^{L/L}$ mice. We evaluated AGTR1 and Nox-4 (nicotinamide adenine dinucleotide phosphate oxidase-4) levels to determine the role of BubR1 in the Ang II response. Results from in vitro assays of renal proximal tubule cells suggest that treatment with small interfering RNA targeting BubR1 suppressed Ang II-induced overexpression of AGTR1. Similarly, the upregulation in Nox4 and Jun N-terminal kinase induced by Ang II administration was repressed by treatment with small interfering RNA targeting BubR1.

Conclusions—Ang II–induced hypertension is caused by AGTR1 overexpression in the kidneys via the upregulation of BubR1 and Nox4. (*J Am Heart Assoc.* 2019;8:e011911. DOI: 10.1161/JAHA.118.011911.)

Key Words: angiotensin II type 1 receptor • budding uninhibited by benzimidazole-related 1 • hypertension • nicotinamide adenine dinucleotide phosphate oxidase-4 • renal proximal tubule cell

Hypertension is a well-known risk factor for vascular conditions such as heart attack, stroke, and chronic kidney disorders.[1-3] Angiotensin II (Ang II) plays a critical role

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in both the development of hypertension and subsequent organ impairment via AGTR1 (angiotensin II receptor type 1).[4] AGTR1 in renal proximal tubule cells (RPTCs) is associated with the development of hypertension.[5] Gurley et al revealed that the abrogation of AGTR1 in the proximal tubule alone is sufficient to lower blood pressure regardless of vascular responses.[5] Removing the Ang II–AGTR1 pathway reduces proximal fluid reabsorption and alters the expression of key sodium transporters, modifying pressure natriuresis. Effective targeting of epithelial functions of the proximal tubule of the kidney may be a useful therapeutic strategy in hypertension. At the molecular level, AGTR1 activation by Ang II can result in DNA damage, leading to cellular senescence.[6] Particularly in RPTCs, the activation of AGTR1 by Ang II can result in the induction of Nox (nicotinamide adenine dinucleotide phosphate oxidase) and subsequent genomic damage, such as a reduction in the size of nuclei, which is a well-known chromosome abnormality.[6-8] AGTR1 is therefore an interesting receptor to study, not only with regard to hypertension but also for its role in cellular senescence. Nevertheless, the relationship between AGTR1 and the

Clinical Perspective

What Is New?

- Systemic mild BubR1 (budding uninhibited by benzimidazole-related 1) reduction attenuated the elevation of systolic blood pressure following angiotensin II (Ang II) administration.
- The attenuating effect on Ang II-induced hypertension by BubR1 reduction is via repression of Ang II receptor type 1 overexpression in the kidney.

What Are the Clinical Implications?

- This study suggests that systemic mild BubR1 reduction represses Ang II-induced hypertension by attenuating Ang II receptor type 1 overexpression in the kidneys.
- This hypotensive effect of BubR1 reduction could be a new strategy for improvement of hypertension.

molecules associated with chromosome abnormalities is not yet fully understood.

Recently, several studies have focused on mice with mutations in mitotic regulators, which cause chromosome instability, because these mice show aging/senescence-related phenotypes.[9,10] BubR1 (budding uninhibited by benzimidazole-related 1) was identified as 1 of the multifaceted kinases of the cell cycle, playing an important role as a mitotic checkpoint for spindle assembly.[11] BubR1 insufficiency in mice has been reported to cause specific early aging-associated systematic phenotypes, including gibbus, short lifespan, cachectic dwarfism, lordokyphosis, cataracts, subcutaneous fat tissue loss, and impaired wound healing.[9,12] Recent studies have reported widely varied roles of the mitotic regulators, including roles in stress signaling. Choi et al revealed that BubR1 and mitotic arrest deficient 2, which is also a spindle checkpoint protein, regulate insulin signaling.[13] Also, the role of Bub1 (budding uninhibited by benzimidazoles-1), which is another spindle checkpoint protein, in transforming growth factor-β signaling, has been reported.[14] These new findings inspired the present study on the relationship between the mitotic regulators and Ang II signaling.

We previously found that Ang II can cause the rapid and robust activation of BubR1 expression in vascular smooth muscle cells.[15] We generated mice with a mild reduction in BubR1 expression (*BubR1*$^{L/L}$ mice), in which BubR1 expression was reduced to 20% of the normal level and senescence-related phenotypes were rarely observed. We additionally found that this mild reduction in BubR1 provided an atheroprotective effect.[16,17] BubR1 insufficiency itself was reported to reduce the number of smooth muscle cells,[9,18] and Kyuragi et al reported that intimal hyperplasia after cervical artery

ligation was decreased in *BubR1*$^{L/L}$ mice via a reduction in the proliferation of vascular smooth muscle cells.[16] Tanaka et al also reported that BubR1 insufficiency attenuated macrophage proliferation and could reduce the incidence of atherosclerotic lesions after cholesterol loading.[17] Despite this progress, how systemic mild BubR1 reduction affects the Ang II response and subsequent organ remodeling has not yet been determined.

In the present study we investigated the role of BubR1 in the Ang II response in *BubR1*$^{L/L}$ mice, with particular focus on the kidneys and renal cells. We first examined the influence of mild BubR1 reduction on blood pressure changes and tissue remodeling after Ang II infusion. Then, to clarify the cause of the different responses to Ang II between mice with and without BubR1 reduction, we examined AGTR1 expression in the kidneys as well as the role of Nox4, which is a key player in the Ang II response.[8]

Materials and Methods

All data, analytic methods, and study materials will be available to other researchers for scientific purposes on reasonable request sent to the corresponding author. All mice in this study were cared for and assessed according to principles outlined in the *Guidelines for the Care and Use of Laboratory Animals* of Kyushu University.

Experimental Animals

BubR1$^{L/L}$ mice were generated by our laboratory as described previously.[16] In short, *BubR1*$^{L/L}$ mice and wild-type littermates (*BubR1*$^{+/+}$) were derived from a mix of 129 and C57BL/6 mice, and BubR1 reduction was performed with electroporation of A pMC1neo cassette into embryonic stem cells, which induced abnormal splicing in intron 5 of the murine *BubR1* gene. Male *BubR1*$^{L/L}$ mice (20–24 weeks old) and age-matched *BubR1*$^{+/+}$ mice were used in all experiments. The reduction of BubR1 expression in *BubR1*$^{L/L}$ mice was checked by genotyping, quantitative real-time polymerase chain reaction, and Western blotting.[16] Experimental protocols and housing facilities were approved by the *Guidelines for the Care and Use of Laboratory Animals* of Kyushu University. (Approval No. 30-207-0).

Ang II Infusion

An osmotic minipump (model 2004; ALZET, Cupertino, CA) containing Ang II (500 ng/kg per minute; Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline was implanted subcutaneously in each mouse[2] under intraperitoneal anesthesia (hydrochloric acid medetomidine, 0.3 mg/kg; midazolam,

4 mg/kg; and butorphanol tartrate, 5 mg/kg). The mice were shaved between the scapulae, where an incision was made to implant the osmotic minipump under the skin. Following implantation, the wound was closed with a 6-0 nylon suture, and the mice were placed in individual cages. The duration of Ang II loading was 7 days.

Blood Pressure Measurement

Mice were fed a regular pelleted diet and housed on a 12-hour light/dark cycle. Their blood pressure was measured daily by noninvasive determination of tail blood volume, flow, and pressure using a volume pressure-recording sensor and an occlusion tail cuff (CODA System; Hakubatec Lifescience Solutions, Tokyo, Japan). This is a highly accurate system with the capability of measuring systolic and diastolic blood pressures and heart rate simultaneously and noninvasively.[19] Before measurement, the mice were placed on a 37°C warming pad until the temperature of the tail-region reached 37°C according to an infrared thermometer. Following warming, the mice were trained for 3 15-minute sessions each day for 7 days or until we obtained stable blood pressure recordings. Blood pressure was measured 45 times every other day; all measurements were performed at the same time of day (10:00–11:30 AM).

Echocardiography

The mice were anesthetized with 1% isoflurane via a nose cone at a flow rate of 0.5 L/min. ECG measurements and respiration were continuously monitored with an integrated physiology platform, and the temperature was maintained at 37°C with a heated platform and ceramic heating lamp. Cardiac function and structural changes were measured using a Vevo 770 ultrasound machine (VisualSonics, Toronto, Canada). Measurements of the thickness of the interventricular septum, left ventricular posterior wall, and left ventricular and anterior walls as well as of the end-diastolic left ventricular internal diameter, end-systolic left ventricular internal diameter, end-diastolic volume, and end-systolic volume were determined using M-mode images in the parasternal long-axis and the left ventricular short-axis views at the midpapillary level.[20,21]

Histological Analyses

Mouse aorta, kidneys, and heart were harvested and incubated in 10% phosphate-buffered formalin overnight, after which samples were excised and embedded in paraffin. Cross sections were processed for hematoxylin/eosin, Elastica van Gieson, Masson trichrome, and Sirius red staining. Four different regions (lumen, intima, media, and total vascular

area) of Elastica van Gieson–stained cross sections were captured with a NanoZoomer-SQ (C13140-L01; Hamamatsu Photonics, Shizuoka, Japan) and analyzed using NDP.view2 (U12388-01; Hamamatsu Photonics). The areas surrounded by the luminal surface, internal elastic lamina, and external elastic lamina were calculated. The intimal area was determined by subtracting the luminal area from the area defined by the internal elastic lamina, and the medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina.

For immunochemistry, all samples were fixed overnight in 10% formaldehyde, embedded in paraffin, and sectioned at a thickness of 3-4 μm. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 in methanol, and nonspecific binding was blocked by incubation with 10% normal goat serum (Nichirei Bioscience, Inc, Tokyo, Japan). Sections were incubated overnight at 4°C with primary antibodies, washed, and incubated with the appropriate secondary antibody for 60 minutes at room temperature. Antigen-antibody complexes were visualized using DAB kits (DAKO; Agilent Technologies, Santa Clara, CA). As a negative control for all experiments, the primary antibody was omitted. Images of the immunostained slides were captured with a NanoZoomer-SQ (C13140-L01; Hamamatsu Photonics). Quantification of all the samples was conducted blindly by calculating the average ratio of positive cells in 10 visual fields under the microscope. The consecutive sections from each animal were graded, and the average used for that individual animal was calculated. Experiments included the following groups: $BubR1^{+/+}$ vehicle control, n=5; Ang II infusion, n=5; $BubR1^{L/L}$ mice vehicle control, n=5; Ang II infusion, n=5.

Immunoblot Analysis

Aorta, heart, and kidney homogenates were prepared in radioimmunoprecipitation assay lysis buffer (Wako, Tokyo, Japan). Samples were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride microporous membranes (Bio Rad, Hercules, CA) and probed with primary antibodies, including monoclonal antibodies against BubR1 (Novus Biologicals, Littleton, CO; NBP1-19555), Agtr1 (Abcam, Cambridge, UK; ab18801), Nox4 (Abcam; ab60940), JNK (Jun N-terminal kinase; Abcam; ab9252), α-tubulin (Abcam; ab4074), and Agtr2 (Santa Cruz Biotechnology; sc-9040). HRP-conjugated antirabbit IgG (1:5000, GE Healthcare UK, Buckinghamshire, UK; NA934V) was used as the secondary antibody. Chemi-Lumi One Ultra (#11644; Nacalai Tesque, Kyoto, Japan) was used for chemiluminescence, and an Amersham Imager 600 (GE Healthcare UK) was used for imaging.
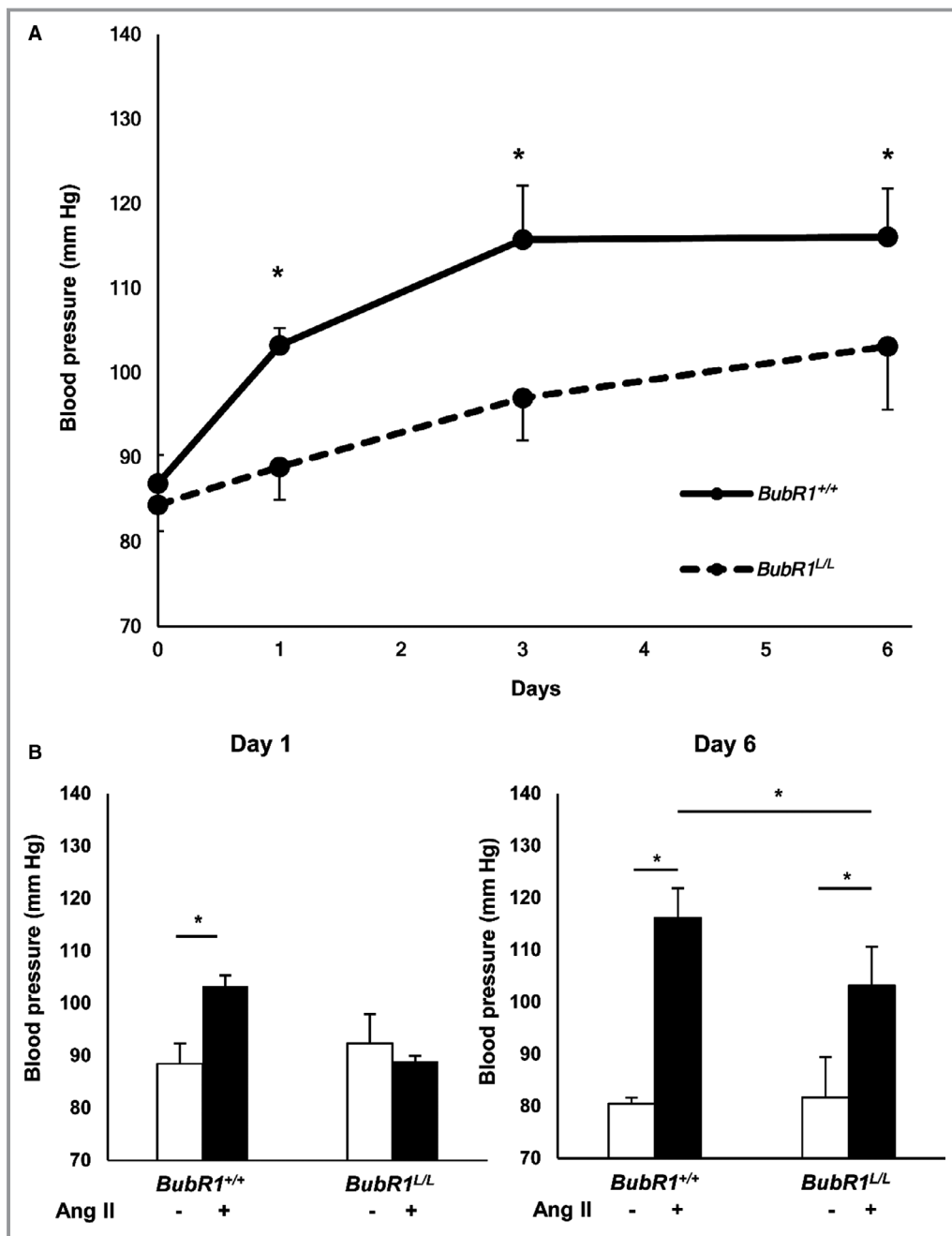
Figure 1. Time-dependent changes in systolic blood pressure (SBP) following angiotensin II (Ang II) infusion in mice. **A**, Time course of SBP levels in Ang II–stimulated BubR1 low-expression (*BubR1^{L/L}*) mice and wild-type (*BubR1^{+/+}*) mice from days 0 to 6 of Ang II infusion. n=10 *BubR1^{+/+}* mice; n=11 *BubR1^{L/L}* mice. **B**, SBP levels at day 1 and day 6 of Ang II infusion. *$P<0.01$ vs *BubR1^{+/+}* mice.

In Vitro Assays

Human RPTCs (Lonza, Basel, Switzerland) were cultured in Smooth Muscle Growth Medium-2 (Lonza) at 37°C with 5% CO_2/95% air. *BubR1* gene silencing was performed using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Inc, Waltham, MA) according to the manufacturer's protocol. RPTCs were transfected with small interfering (si)RNA targeting *BubR1* (siBubR1) (sc-37542; Santa Cruz Biotechnology) or the scrambled siRNA control (sc-37007; Santa Cruz Biotechnology) at a final concentration of 10 nmol/L in transfection reagent (1:500; vol/vol). Twenty-four hours after siRNA transfection, siRNA-mediated RPTCs were treated with 10 μmol/L Ang II for 24 hours and collected for analysis.

In the cells, protein expression was analyzed by Western blots. Each cell's homogenates were prepared in
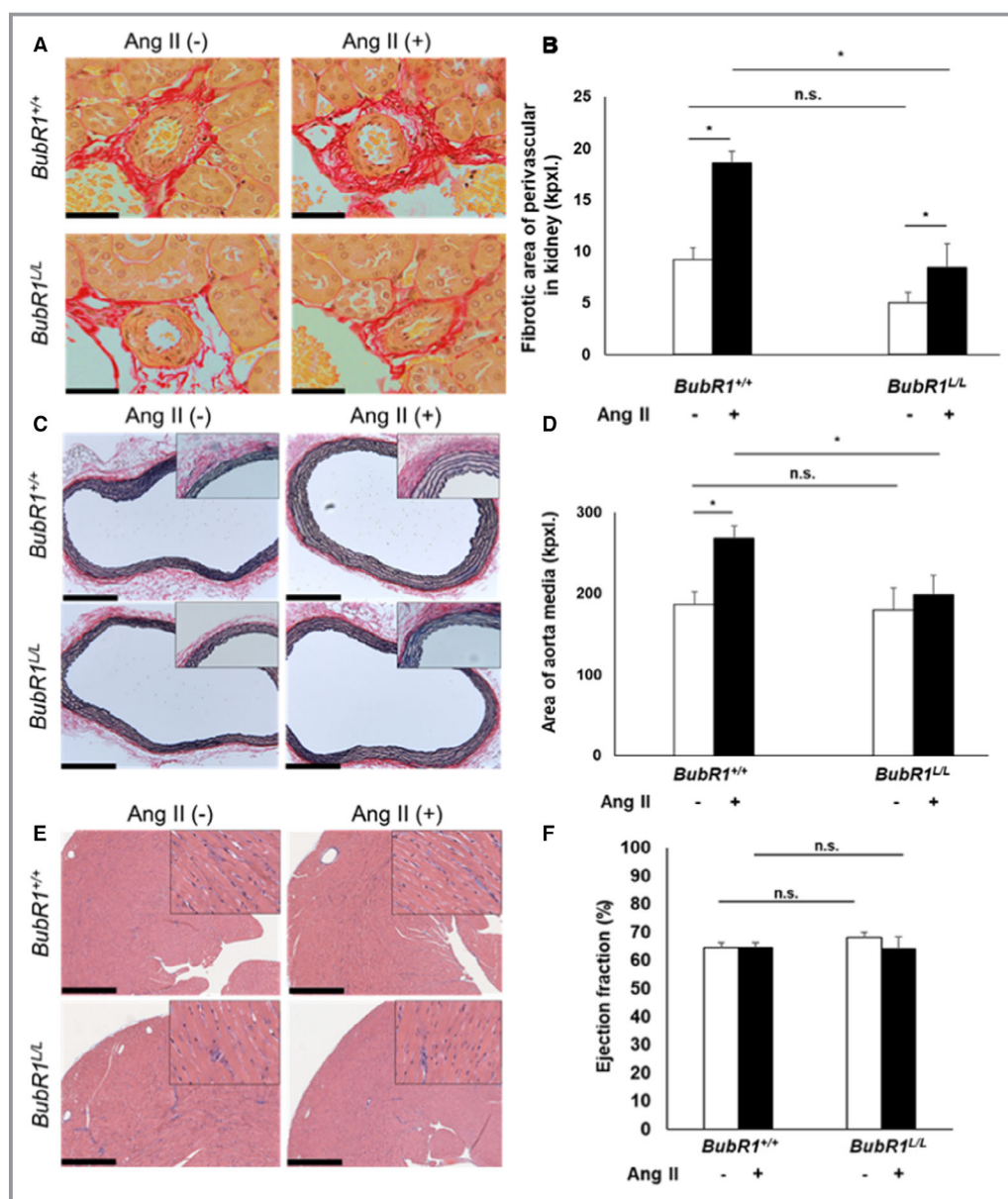
Figure 2. Histological analysis of tissues from BubR1 low-expression (*BubR1*$^{L/L}$) mice and wild-type (*BubR1*$^{+/+}$) mice after 1 week of angiotensin II (Ang II) infusion. **A**, Representative Sirius red–stained kidney sections. Magnification, ×40; n=10 *BubR1*$^{+/+}$ mice; n=8 *BubR1*$^{L/L}$ mice; scale bar, 100 μm. **B**, Perivascular fibrotic lesion area in the kidney, quantified using morphometry. *P<0.01 vs *BubR1*$^{+/+}$ mice. **C**, Representative Elastica van Gieson–stained sections of the thoracic aorta. Magnification, ×40; n=12 *BubR1*$^{+/+}$ mice; n=11 *BubR1*$^{L/L}$ mice; scale bar, 100 μm. **D**, Area of aorta media, quantified using morphometry. *P<0.01 vs *BubR1*$^{+/+}$ mice. **E**, Representative Masson trichrome–stained sections of the heart. Magnification, ×40; n=12 *BubR1*$^{+/+}$ mice; n=11 *BubR1*$^{L/L}$ mice; scale bar, 100 μm. **F**, Ejection fraction, evaluated by echocardiographs; n=12 control mice; n=11 *BubR1*$^{L/L}$ mice. *P<0.01 vs *BubR1*$^{+/+}$ mice.

radioimmunoprecipitation assay lysis buffer (Wako, Tokyo, Japan). Samples were subjected to SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride microporous membranes (Bio Rad, Hercules, CA) and probed with primary antibodies; monoclonal antibodies against BubR1 (Novus Biologicals; NBP1-19555), Agtr1 (Abcam; ab18801), Nox4 (Abcam;

ab60940), JNK (Abcam; ab9252), α-tubulin (Abcam; ab4074), or Agtr2 (Santa Cruz Biotechnology; sc-9040). HRP-conjugated antirabbit IgG (1:5000, GE Healthcare UK; NA934V) was used as the secondary antibody. Chemi-Lumi One Ultra (#11644; Nacalai Tesque) was used for chemiluminescence, and an Amersham Imager 600 (GE Healthcare UK) was used for imaging.
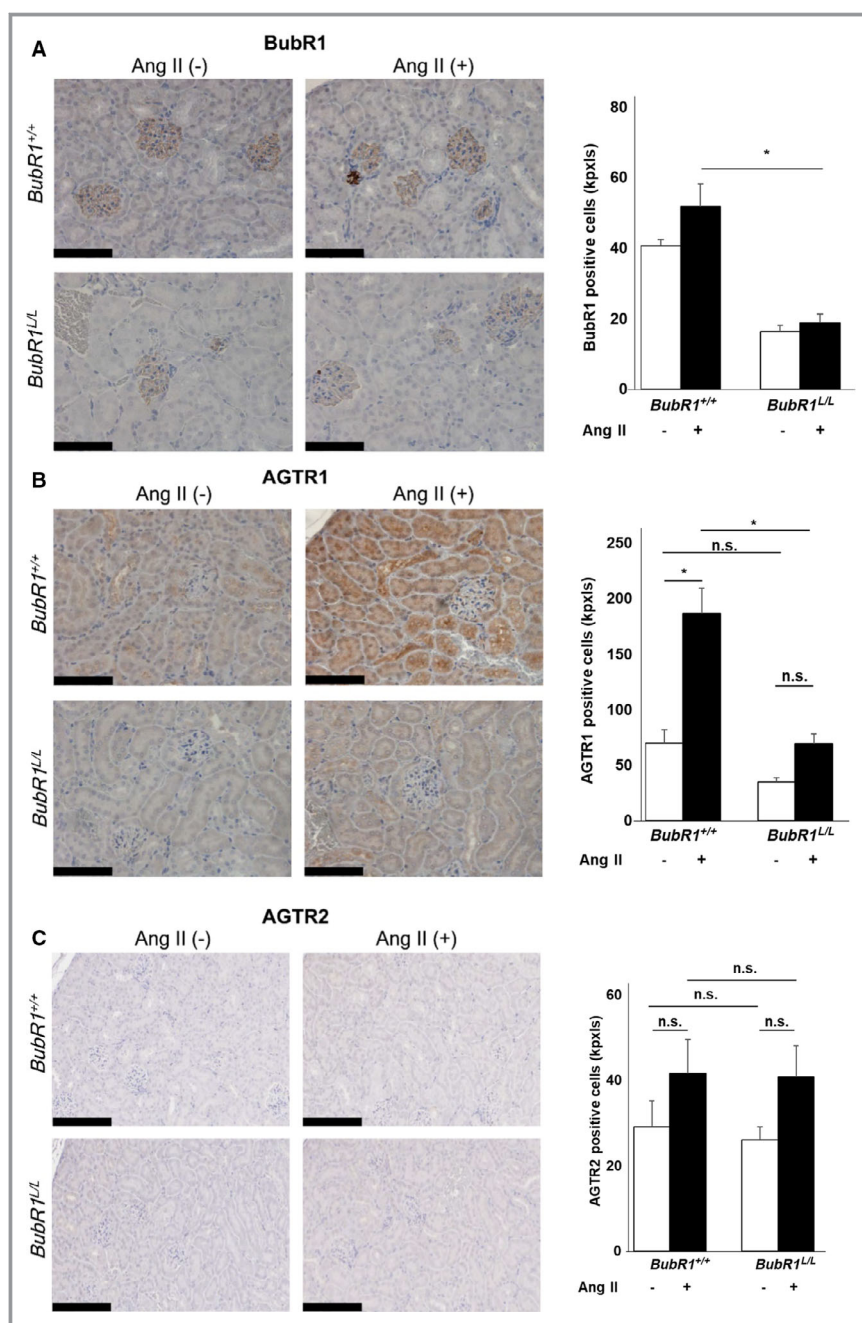
Figure 3. Immunohistochemistry of kidneys from $BubR1^{+/+}$ and $BubR1^{L/L}$ mice before and after 1 week of angiotensin II (Ang II) infusion. **A** through **C**, Images of representative kidney sections that were immunostained for BubR1 (**A**), Ang II receptor type 1 (AGTR1) (**B**), or Ang II receptor type 2 (AGTR2) (**C**). Magnification, ×40; scale bar, 50 μm. BubR1, AGTR1, and AGTR2 expression levels in kidney with or without BubR1 reduction, with or without angiotensin II (Ang II) stimulation (n=5/group). *$P<0.01$. kpxls indicates kilo pixels; n.s., not significant.

Statistical Analyses

Data are expressed as mean±standard error. The statistical significance of differences between groups was determined using the Wilcoxon rank sum test. All analyses were performed using JMP software (ver 11.0; SAS Institute, Cary, NC). A P-value of <0.01 was considered statistically significant.

Results

Systolic Blood Pressure Following Ang II Infusion in $BubR1^{L/L}$ Mice and $BubR1^{+/+}$ Mice

To clarify the role of BubR1 in the Ang II response, we generated $BubR1^{L/L}$ mice in which the BubR1 expression

was reduced to 20% of the normal level (Figure S1). BubR1 downregulation alone did not cause a change in the systolic blood pressure before Ang II infusion in each mouse of Ang II infusion groups (Figure 1A). At day 1 of Ang II infusion, $BubR1^{L/L}$ mice showed a lack of Ang II–induced hypertension, which was already detectable in $BubR1^{+/+}$ mice (Figure 1B) in response to Ang II administration. The systolic blood pressure in $BubR1^{L/L}$ mice increased to 101.5±13.2 mm Hg after 6 days of Ang II administration, but this value was significantly lower than that in similarly treated $BubR1^{+/+}$ mice (119.8±12.1 mm Hg) ($P<0.01$; Figure 1A). The systolic blood pressure at 3 days after vehicle administration instead of Ang II administration did not increase in either $BubR1^{+/+}$ or $BubR1^{L/L}$ mice (Figure S2).

Ang II–Induced Tissue Remodeling

To further investigate the different blood pressure responses to Ang II between $BubR1^{+/+}$ and $BubR1^{L/L}$ mice, we evaluated tissue remodeling of the kidney, aorta, and heart. Ang II induced perivascular fibrosis in the kidneys in both types of mice, although there were significantly fewer fibrotic lesions in $BubR1^{L/L}$ mice than in $BubR1^{+/+}$ mice (Figure 2A and 2B). Similarly, the aortic media increased in both types of mice after 1 week of Ang II infusion, but the level of increase in $BubR1^{L/L}$ mice was significantly attenuated compared with

that in $BubR1^{+/+}$ mice ($P<0.01$; Figure 2C and 2D). Ang II treatment also induced a slight fibrosis in the heart, but there was no difference between $BubR1^{+/+}$ and $BubR1^{L/L}$ mice (Figure 2E and Figure S3). To assess whether BubR1 insufficiency caused functional changes in the heart after Ang II infusion, echocardiography was performed, and the ejection fraction was evaluated (Figure S4). In agreement with the histological findings, the ejection fractions in $BubR1^{+/+}$ and $BubR1^{L/L}$ mice after Ang II infusion were similar to one another, showing no significant difference (Figure 2F).

Ang II–Induced AGTR1 and AGTR2 Expression in Kidneys

Our histological analysis suggested that the BubR1 reduction largely attenuated Ang II–induced fibrosis in the kidneys rather than in the heart. Notably, Ang II–induced hypertension in the early phase is caused mainly by a reaction in the kidneys.[22]

To investigate causes for the reduction in Ang II–induced hypertension in $BubR1^{L/L}$ mice, we examined the AGTR1 and AGTR2 (angiotensin II receptor type 2) expression in the kidneys after Ang II infusion. Figure 3A through 3C shows the results of an immunohistochemical examination of BubR1, AGTR1, and AGTR2, respectively. BubR1 was mainly present in the renal glomeruli in both types of mice,
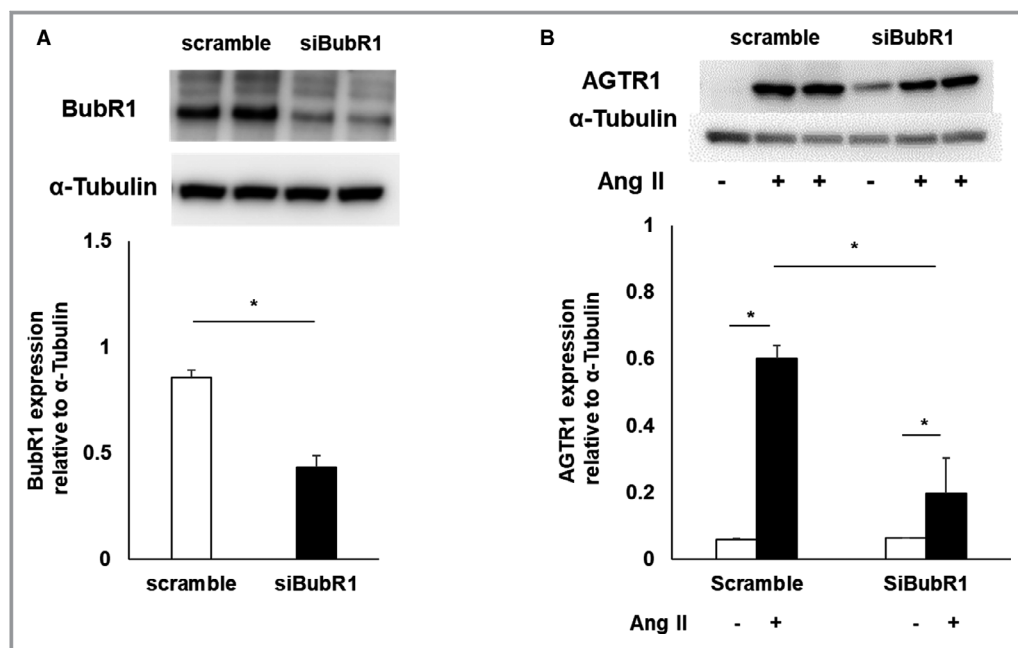

Figure 4. In vitro assay of BubR1 reduction in human renal proximal tubule cells (RPTCs). **A**, BubR1 levels in RPTCs that were treated with siBubR1 or scrambled control siRNA (3 biological replicates each). *$P<0.01$. **B**, Angiotensin II (Ang II) receptor type 1 (AGTR1) expression levels in RPTCs with or without BubR1 reduction, with or without 10 μmol/L Ang II stimulation for 24 hours (n=3/group). *$P<0.01$. siBubR1 indicates short interfering RNA targeting *BubR1*.

with *BubR1*$^{L/L}$ mice showing the expected lower BubR1 levels compared with *BubR1*$^{+/+}$ mice (Figure 3A). Immuno-histochemical examination revealed that the AGTR1 expression increased in both types of mice after Ang II infusion but that the AGTR1 expression in *BubR1*$^{L/L}$ mice was lower than that in *BubR1*$^{+/+}$ mice (Figure 3B). There was no significant difference in AGTR2 levels between *BubR1*$^{L/L}$ and *BubR1*$^{+/+}$ mice (Figure 3C). These results suggest that the BubR1 reduction in the kidneys of *BubR1*$^{L/L}$ mice repressed the AGTR1 overexpression that is otherwise induced by Ang II.

Ang II–Induced AGTR1 Expression in RPTCs

To determine how a reduction in BubR1 levels attenuates the Ang II–induced AGTR1 upregulation in the kidneys, we used a RPTC in vitro assay because the Ang II–AGTR1 reaction in RPTCs is the most important cause of hypertension development.[5] BubR1 downregulation was performed via siRNA transfection. RPTCs treated with siBubR1 showed an ≈40%

reduction in BubR1 expression (Figure 4A). The AGTR1 expression was similar between control and siBubR1-treated RPTCs. After Ang II stimulation, the AGTR1 expression in both control and siBubR1-treated cells significantly increased, but the siBubR1-treated RPTCs showed a significantly lower response compared with the control-treated cells (Figure 4B). These results suggest that BubR1 affects Ang II–induced AGTR1 upregulation at the cellular level in the kidneys.

Ang II–Induced Nox4 and JNK Expression

AGTR1 overexpression in the kidneys is activated by Nox.[23] We next investigated how BubR1 affects Nox4 during Ang II stimulation in RPTCs. Figure 5B shows the expression changes of Nox4 and JNK that occurred during Ang II stimulation with or without BubR1 reduction. The Ang II–induced increase in Nox4 levels was attenuated in siBubR1-treated cells relative to that in cells with ordinary BubR1 expression ($P<0.01$). BubR1 downregulation also
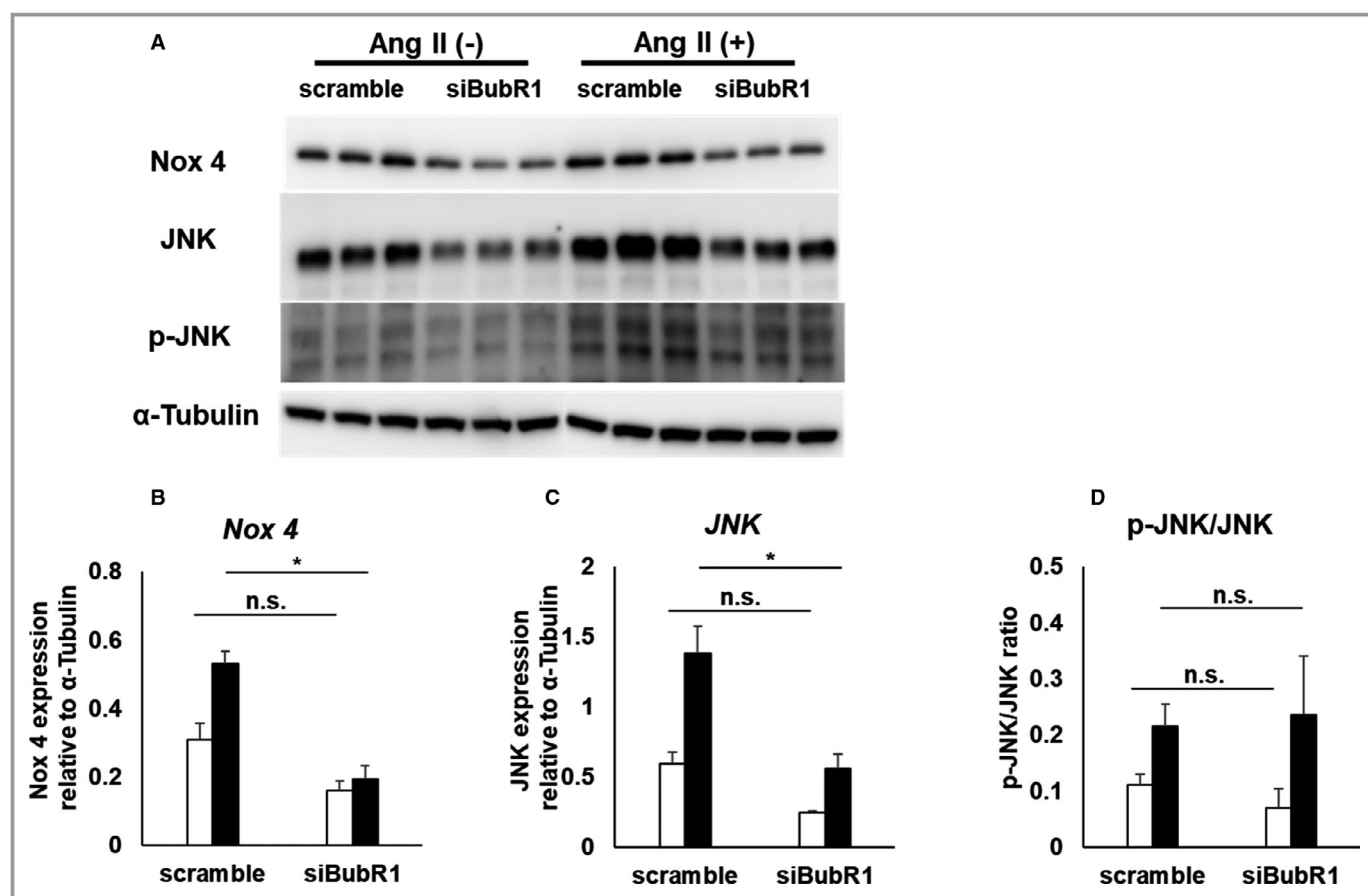

Figure 5. Expression levels of Nox 4, JNK, and phospho-JNK in human renal proximal tubule cells (RPTCs), with or without angiotensin II (Ang II) stimulation. **A**, Representative Western blot of Nox 4, JNK, and phospho-JNK/JNK in siBubR1- or scramble siRNA control–treated RPTCs, with or without Ang II stimulation. **B** through **D**, Quantified expression levels of Nox 4 (**B**), JNK (**C**), and phospho-JNK/JNK (**D**) from the blot in (**A**) (n=6/group). *$P<0.01$. JNK indicates Jun N-terminal kinase; Nox4, nicotinamide adenine dinucleotide phosphate oxidase–4; n.s., not significant; p-JNK, phospho-JNK; siBubR1, short interfering RNA targeting *BubR1*.
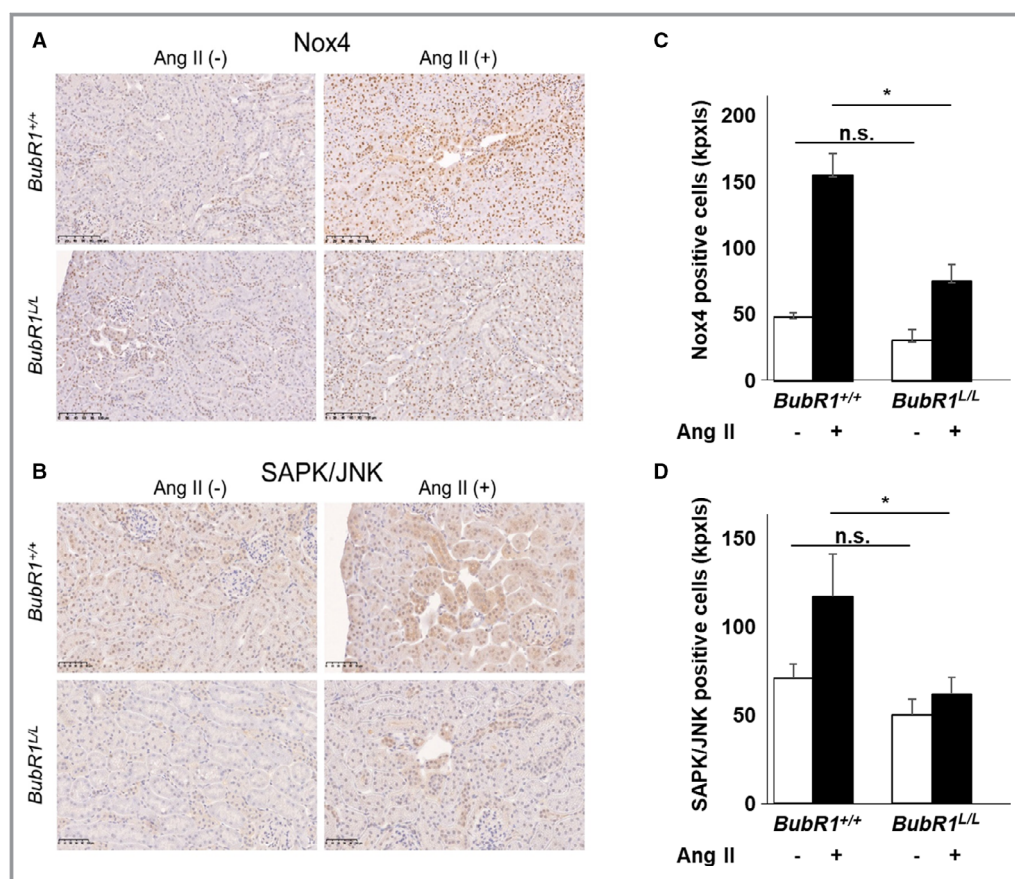
Figure 6. Immunohistochemistry of kidneys from $BubR1^{+/+}$ and $BubR1^{L/L}$ mice before and after 1 week of angiotensin II (Ang II) infusion. Representative immunohistochemistry sections of Nox4 (**A**) and JNK (**B**) expression. Scale bar, 100 μm. Nox4 and SAPK/JNK expression levels in kidney with or without BubR1 reduction, with or without Ang II stimulation (n=5/group). Quantified expression levels of Nox 4 (**C**), and SAPK/JNK (**D**). *$P<0.01$. JNK indicates Jun N-terminal kinase; Nox4, nicotinamide adenine dinucleotide phosphate oxidase-4; n.s., not significant; SAPK, stress-activated protein kinase.

attenuated the increases of amount of JNK (Figure 5C), whereas there was no significant difference in the phospho-JNK/JNK ratio between the 2 groups (Figure 5D). These results suggest that BubR1 downregulation repressed both Nox4 and total JNK expression but not JNK phosphorylation.

In agreement with the above in vitro data, the results of an immunohistochemical examination showed that the overexpression of Nox4 and JNK observed in the kidneys of $BubR1^{+/+}$ mice after Ang II infusion was significantly repressed in the kidneys of Ang II–stimulated $BubR1^{L/L}$ mice (Figure 6).

Discussion

We found that systemic mild BubR1 reduction attenuated Ang II–induced hypertension by repressing Ang II–induced AGTR1 overexpression in the kidneys of $BubR1^{L/L}$ mice. BubR1 reduction also repressed the upregulation of Nox4 that is otherwise induced by Ang II stimulation in RPTCs.

Ang II plays a key role in hypertension by controlling blood pressure and volume in the cardiovascular system through at least 2 types of receptors, AGTR1 and AGTR2. AGTR1 has hypertensive roles, and AGTR2 has hypotensive roles.[4] When Ang II binds to AGTR1, the volume of circulating plasma increases, and peripheral vascular smooth muscle contracts, resulting in hypertension.[24] When Ang II binds to AGTR2, the resulting hypotension is due to vasodilation.[25] Thus, the 2 angiotensin receptors have contradictory roles and compete with each other.[26] Ang II induces hypertension mainly by increasing the volume of circulating plasma, which is controlled in the kidneys.[27,28] For blood pressure regulation, an appropriate balance between fluid intake and renal excretion is required. Blood pressure is also influenced by several vasoactive factors that regulate nephron transport. An imbalance in the regulation of sodium reabsorption by nephrons contributes to hypertension.[29] For renal hypertension, Ang II provokes AGTR1 overexpression, and AGTR1 functions in the development of hypertension.

In the current study we found that systemic mild BubR1 reduction repressed the Ang II–induced elevation of systolic blood pressure (Figure 1A). This repression was observed at an early stage of Ang II infusion (Figure 1B). Fibrosis in the kidney was also attenuated in *BubR1^{L/L}* mice (Figure 2A). These results suggest that the repression of Ang II–induced hypertension was caused mainly by a weakened response to Ang II in the kidneys of *BubR1^{L/L}* mice. Supporting this idea is the finding that the Ang II–induced AGTR1 expression was significantly decreased in the kidneys of *BubR1^{L/L}* mice compared with *BubR1^{+/+}* mice (Figure 3B). Furthermore, in the absence of Ang II infusion, there was no difference in the AGTR1 expression in the kidney between *BubR1^{+/+}* mice and *BubR1^{L/L}* mice. Therefore, BubR1 seems to play a critical role in Ang II–induced hypertension by controlling the AGTR1 expression in the kidney.

We also observed an attenuation of aortic medial hyperplasia in *BubR1^{L/L}* mice by day 7 of Ang II stimulation (Figure 2B). Repressed hypertension can reduce aortic medial hyperplasia.[30,31] Additionally, BubR1 insufficiency itself inhibits cell proliferation[15] and makes vascular smooth muscle cells unprogressive.[16,18] Thus, the observed attenuation of aortic medial hyperplasia in *BubR1^{L/L}* mice is likely the result of both repressed hypertension and systemic BubR1 insufficiency in these mice rather than the cause of the attenuated Ang II–induced hypertension.

Cardiac function is important for maintaining normal blood pressure. However, no histological or functional changes in the heart corresponding with the BubR1 reduction were observed in the current study (Figure 2C and 2D). This could be because there is intrinsically very little BubR1 expression in cardiomyocytes (Figure S3), and a reduction in BubR1 could not arise from biological changes in the heart. Furthermore, Ang II may have very little effect on cardiomyocytes, especially in the early phase of Ang II infusion.[32] Regardless of the limited contributions of BubR1 to Ang II–induced hypertension in the aorta and heart, the change in BubR1 expression in the kidneys seems to play an important role in repressing Ang II–stimulated blood pressure elevations.

BubR1 affected the AGTR1 expression in the kidneys. BubR1 seemed mainly present in the glomeruli (Figure 3A); however, the AGTR1 expression was suggested to be quite small,[33] and we could not detect the influence of BubR1 on glomerular endothelial cells (Figure S5). Conversely, AGTR1 in proximal tubules is known to play a critical role in controlling blood pressure regardless of vascular responses.[5] We assessed how BubR1 affects the Ang II response of RPTCs and found that a BubR1 reduction remarkably attenuated the Ang II–induced AGTR1 upregulation in RPTCs (Figure 4B). After this observation, we further investigated the molecular relationship between BubR1 reduction and AGTR1 overexpression. Ang II stimulates the generation of Nox4 in the

kidneys through AGTR1 activation,[8] and Nox4 is an important factor in hypertension. Ang II–induced Nox4 upregulation is a known source of oxidative stress and can cause organ failure.[23,34] Additionally, Nox4 is present in kidney and vascular cells[35,36] and is required to mediate Ang II–induced protein synthesis.[37] Ang II activates JNK signaling through Nox4, resulting in AGTR1 expression.[38,39] In agreement with these previous findings, we found that BubR1 insufficiency attenuated the Ang II–induced upregulation of Nox4, JNK, and phospho-JNK (Figure 5). JNK signaling exists between Nox4 and AGTR1, and phospho-JNK activates the transcription of AGTR1. Here, we found that BubR1 insufficiency reduced the total amount of JNK (Figure 5C); however, the JNK phosphorylation rate was not different between control and siBubR1-treated cells (Figure 5D). These results suggest that BubR1 insufficiency affected the expression, but not the phosphorylation, of JNK. Together, our findings suggest that a reduction in BubR1 represses the Ang II stimulation-induced AGTR1 overexpression in the kidneys by attenuating JNK and Nox4 upregulation.

In summary, a systemic mild BubR1 reduction repressed Ang II-induced hypertension by attenuating AGTR1 overexpression in the kidneys. The hypotensive effect of BubR1 reduction in RPTCs occurred through a repression of the upregulations of AGTR1 and Nox4. Renal-specific BubR1 reduction may be a promising new therapy for preventing the development of hypertension and AGTR1 overexpression in the kidneys by acting as an AGTR1 blocker. This would be useful not only as an antihypertensive therapy but potentially also as a means of protecting heart and kidney functions.[37–39] Further studies are required to clarify the relationship between BubR1 and the Ang II–AGTR1 pathway.

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Disclosures

None.

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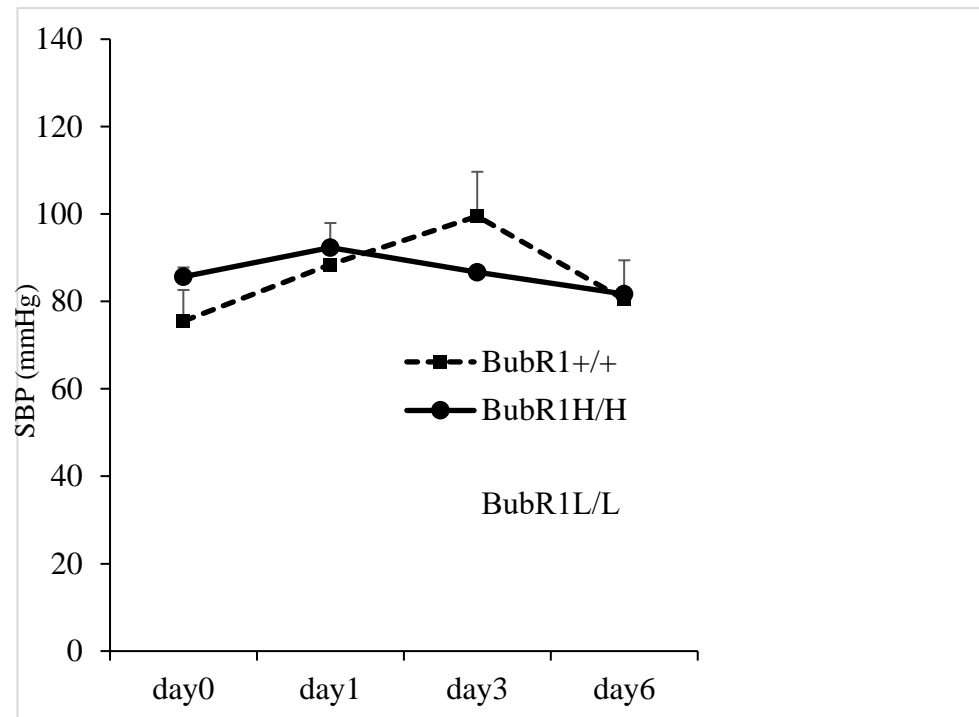
Supplemental Material

Figure S1. Protein expression levels of BubR1 in thymus of BubR1$^{+/+}$ and BubR1$^{L/L}$ mice.
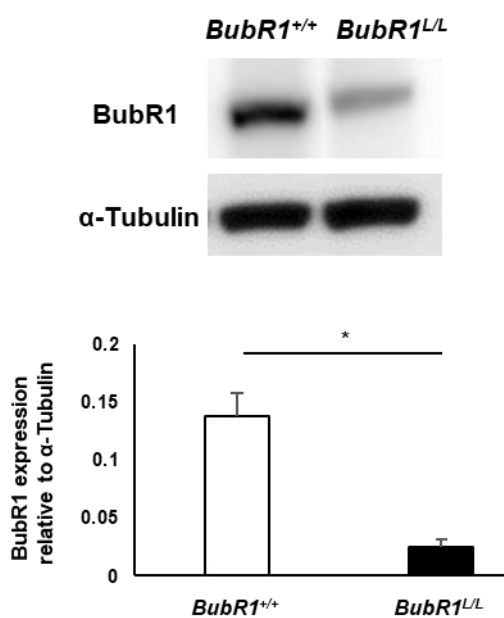

*P < 0.01.

Figure S2. Only BubR1 downregulation was not affected in systolic blood pressure.

Time-dependent change of systric blood pressure in mice (Tail-cuff procedure).

There was no significant differene of systolic blood pressure in either genotypes without

angiotensin II administration. n=6 per group.

**Figure S3. Immunohistochemical staining of BubR1 in heart of both mice.**
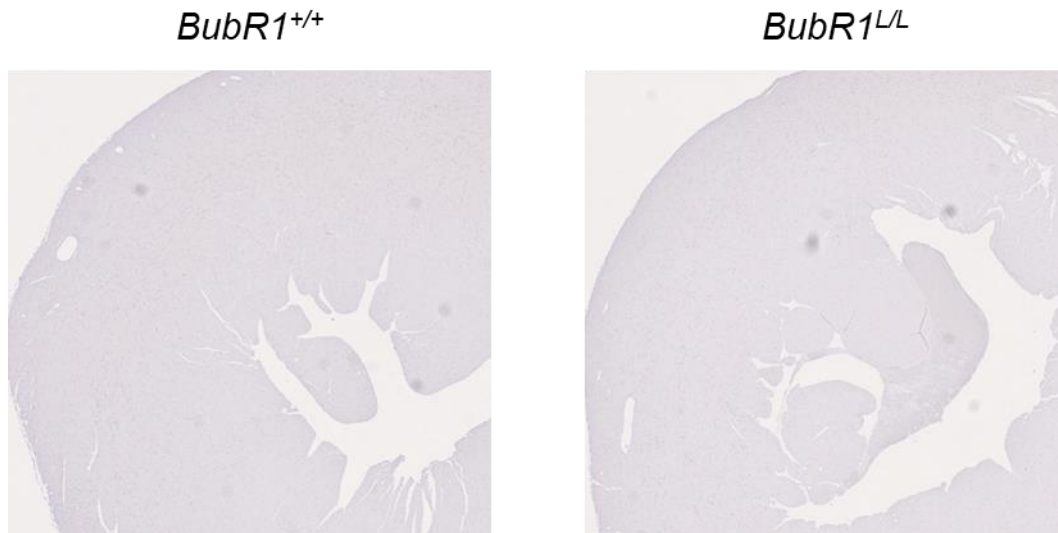
BubR1 expression of heart was little in both groups. Representative immunohistochemistry

sections taken at 40× of murine Heart from BubR1^{+/+} and *BubR1[L/L]* mice (n=4 BubR1^{+/+}

mice; n=5 *BubR1[L/L]* mice).

Figure S4. Representative images of echocardiographs.
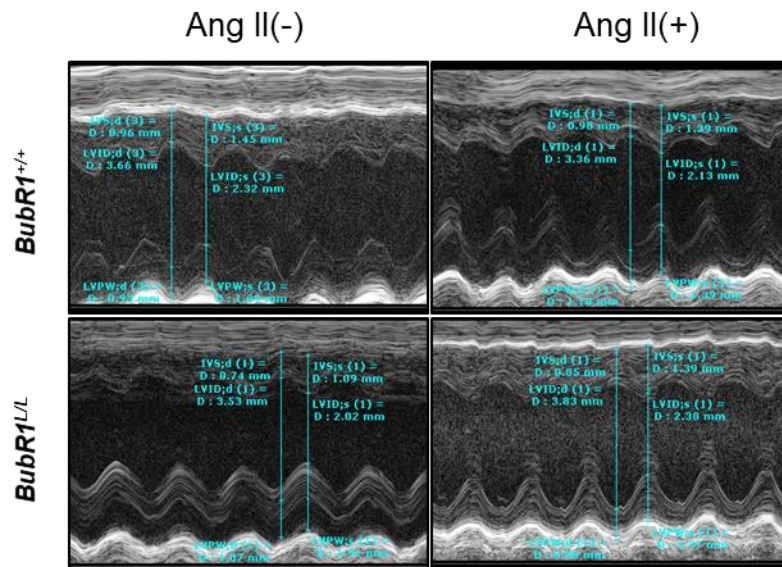
There are no differences about cardiac output, ejection fraction and fractional shortening at

both mice after a week angiotensin II infusion (n=12 $BubR^{+/+}$ mice; n=11 $BubR1^{L/L}$ mice).

Figure S5. Agtr1 expression in human renal glomerular endothelial cell.
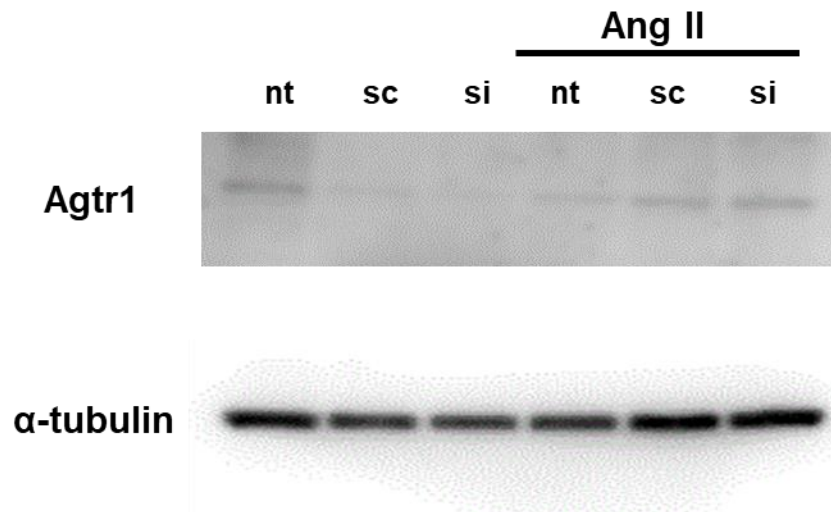
Protein expression levels of Agtr1 in human renal glomerular endothelial cell. Protein

expression was measured via Western blotting. (nt=non treatment, sc=scramble, si=siBubR1)